Virology 484 (2015) 15–21

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

# Amino acid substitution D222N from fatal influenza infection affects receptor-binding properties of the influenza A(H1N1)pdm09 virus

Adriana Matos-Patrón [a], Lauren Byrd-Leotis [b], David A. Steinhauer [b], Wendy S. Barclay [c], Guadalupe Ayora-Talavera [a,*]

[a] Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autonoma de Yucatan, Av. Itzaes #490 × 59, Centro, C. P., 97000 Merida, Yucatan, Mexico
[b] Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322, USA.
[c] Section of Virology, Faculty of Medicine, Imperial College London, London, United Kingdom

ARTICLE INFO

*Article history:*
Received 7 February 2015
Returned to author for revisions
15 March 2015
Accepted 12 May 2015
Available online 5 June 2015

*Keywords:*
Influenza
Receptor
Binding
A(H1N1)pdm09
Mexico

ABSTRACT

We have analyzed the receptor binding profile of A(H1N1)pdm09 recombinant influenza viruses containing the amino acid substitution D222N which has been associated with a fatal case of infection. This mutation was investigated in conjunction with a secondary mutation, S185N. Using human tracheobronchial epithelial cells (HTBE), we found that single mutation D222N affects the binding and replication of the virus during initial stages of infection, with limited but preferred tropism to non-ciliated cells expressing α2,6-SA. However, in conjunction with the S185N change, the (D222N, S185N) virus shows a remarkable increase in binding and replication efficiency, with tropism for both ciliated and non-ciliated cells. Glycan microarray analysis demonstrated correlation between the binding profile and the cell tropism observed in the HTBE cells. These findings suggest that viruses with D222N required compensatory mutations such as S185N to maintain viral fitness, and in combination, affect the pathogenicity of the virus and the clinical outcome.



## Introduction

The 2009 influenza pandemic in Mexico was characterized by an unusual increase in the number of severe and fatal respiratory infections associated with the isolation of the novel influenza A (H1N1)pdm09 virus (Chowell et al., 2009). The influenza outbreak in southern Mexico between June and August of that year was considered the second wave of the pandemic, with the highest number of infections reported in Yucatan (Chowell et al., 2011). During this time and in the following months, the health authorities in Yucatan recorded a total of 92 cases of severe or fatal influenza infection (Ayora-Talavera et al., 2012). Molecular markers of pathogenicity, including amino acid changes mapping to the receptor binding site (RBS) of the viral hemagglutinin (HA) were found in some of the patients with severe or fatal influenza infections. Isolates of the new A(H1N1)pdm09 virus containing a D222G amino acid change in the RBS were reported in different geographic regions at frequencies varying from 5% up to 24% (Chen et al., 2010; El Moussi et al., 2013; Ledesma et al., 2011; Rykkvin et al., 2013; Vazquez-Perez et al., 2013). The pathogenic effects of viruses with the D222G mutation have been analyzed in mice, Guinea pigs, and ferrets, but the results and conclusions differ depending on the study and animal model utilized (Abed et al., 2011; Casalegno et al., 2014; Chutinimitkul et al., 2010; Vazquez-Perez et al., 2013).

The genetic polymorphism 222D/G/N in HA amino acid 222 of the A(H1N1)pdm09 virus has been identified in circulating viruses, and for virus populations within the same host a higher frequency of mutants with G, N, or both, is suggested to relate to the severity of the infection (Baldanti et al., 2011; Resende et al., 2014). Interestingly, the D222N mutation has generally been detected in combination with D or G heterogeneity at this position, and in only few reports as the major population (Baldanti et al., 2011; Casalegno et al., 2014; Drews et al., 2011; Resende et al., 2014; Wang et al., 2011). The presence of any polymorphism at residue 222 may confer changes to the virus receptor binding profile and the cell/tissue tropism, as D222G HA mutants of A(H1N1)pdm09 virus showed a preference for infection of ciliated cells from HTBE (Chutinimitkul et al., 2010), consistent with α2,3-SA specificity (Matrosovich et al., 2004; Thompson et al., 2006), and with changes in the tissue tropism as demonstrated for the highly pathogenic H5N1 virus (Shinya et al., 2006; Van Riel, et al., 2006). However, the A(H1N1)pdm09 virus with D222G also retained ability to infect non-ciliated cells expressing α2,6-SA, showing dual receptor

* Corresponding author.
*E-mail address:* talavera@correo.uady.mx (G. Ayora-Talavera).

http://dx.doi.org/10.1016/j.virol.2015.05.012
0042-6822/

specificity, as well as dual binding to α2,3-SA and α2,6-SA glycans (Chutinimitkul et al., 2010; Liu et al., 2010; Yang et al., 2010).

A(H1N1)pdm09 viruses with the HA D222N substitution have also been isolated and characterized since 2009 (Wang et al., 2011), and viruses with this mutation have been detected during outbreaks of severe and fatal influenza in Mexico, Ecuador, and the U.S. Interestingly, these cases were associated with a prominent D222N and not the polymorphic trait of past isolates. Phylogenetic analysis identified a new sub-clade containing additional amino acid changes within the HA: N31D, S162N, A186T and V272I (Houng et al., 2012). The role of these mutations on the biology of the pandemic virus is unknown.

Although the glycan binding profiles of both natural and recombinant A(H1N1)pdm09 viruses containing the mutation D222N have been reported (Bradley et al., 2011; Chen et al., 2011), the precise role of the amino acid change D222N on the severity of the infection is unclear. In this study, we address further the receptor binding phenotype of A(H1N1)pdm09 viruses with polymorphisms 222D/N, and the effect on the cell tropism using the in vitro system of human airway epithelial cells.

## Results and discussion

*Mutation D222N affects the early replication of A(H1N1)pdm09 virus in HTBE cultures but is compensated by secondary mutations in the antigenic site Sb*

In order to identify mutations in the HA RBS that might affect binding phenotype and pathogenicity, we performed sequence analysis of HA genes from 13 viral isolates from severe and fatal cases from Yucatan, Mexico. From this, relevant mutations were identified in only two viruses, A/Yucatan/5783/2009 (Yuc/5783) and A/Yucatan/3882/2009 (Yuc/3882). The Yuc/5783 virus was isolated from the bronchial lavage of a hospital patient with a severe case of Influenza. This virus contained a single mutation, D190Y, at the HA protein. The effect of this mutation on the receptor binding properties of the virus was recently described by our group (Ayora-Talavera et al., 2014). The virus Yuc/3882, isolated by throat swab from patient that subsequently died from the infection, contained two mutations in the receptor binding region of HA, S185N and D222N. Due to the relevance of the mutation D222N and the lack of data about its specific role on the pathogenicity of the pandemic virus, we examined the receptor binding profile and replication properties of viruses containing this mutation. We also addressed the relevance of the mutation S185N, located at the antigenic site Sb (Xu et al., 2010). A different amino acid change, S185T, is reported to be conserved in all A(H1N1)pdm09 viruses in current circulation, and its role in the enhancement of receptor-binding avidity of the early A(H1N1)pdm09 virus has been addressed (de Vries et al., 2013; ECDC 2013; Elderfield et al., 2014; Klimov et al., 2012).

Therefore we generated a series of mutants based on the United Kingdom prototype A(H1N1)pdm09 virus, A/England/195/2009 (Eng/195), altered only in the HA gene (Baillie et al., 2012). Each mutant virus contained either amino acid substitution S185N (rEng/195-S185N), D222N (rEng/195-D222N) or S185N+D222N (rEng/195-S185N+D222N). Sequence analysis and comparison between the Eng/195 and Yuc/3882 viruses indicated a 99.7% homology for the HA protein with only three amino acid differences S185N, S203T and D222N.

The growth properties of the recombinant mutant viruses were compared to recombinant Eng/195 (rEng/195-wt) in differentiated pseudostratified human tracheal–bronchial epithelial cells (HTBE, MatTek – Massachusetts, USA). Mutants with S185N whether as a single mutant, or in combination with D225N (rEng/195-S185N and rEng/195-S185N+D222N), replicated to higher titers at early time points than did the wild type virus rEng/195-wt (Fig. 1A). At 12 h post-infection (h.p.i.) the two mutants with S185N already displayed recoverable virus, whereas rEng/195-wt virus did not, and at 24 h, the higher titers for the two mutants were significant ($p < 0.0001$). The slow replication of the rEng/195-wt virus is probably due to differences in adaptation to the HTBE system; our data are in agreement with recent results that showed a delayed viral growth for the rEng/195-wt used as prototype virus from the first pandemic wave compared to a recombinant virus from the third wave (Baillie et al., 2012; de Vries et al., 2013; Elderfield et al., 2014). Similar to the wt, the rEng/195-D222N showed null viral growth at 12 h.p.i. ($p < 0.0001$), although at 24 h.p.i. and at later timepoints, the virus reached a maximum viral titer similar to the other two mutants. Overall, these results suggest that the presence of mutation D222N alone has a negative effect on the viral replication during the initial rounds of replication. However, the presence of mutation S185N alone, or in combination with D222N, correlated with faster replication of these viruses in HTBE cells at early time points.

In MDCK cells the growth curve (Fig. 1B) showed that rEng/195-wt virus replicated most efficiently. Significant differences were observed between the rEng/195-wt viral titer versus rEng/195-S185N at 12 ($p < 0.01$), and rEng/195-222N and rEng/195-S188N+D222N ($p < 0.0001$) at 24 h post-infection. The rEng/195-S185N virus displayed higher titers at 24 h than the other two mutants. These data suggest that in MDCK cells, recombinant viruses did not show replication constraints, even in the presence of specific mutations. The delayed viral replication we previously observed for the rEng/195-wt it was likely to be associated with the complex HTBE culture system, a feature that allows the rEng/195-D222N virus to replicate efficiently after 24 h post-infection.

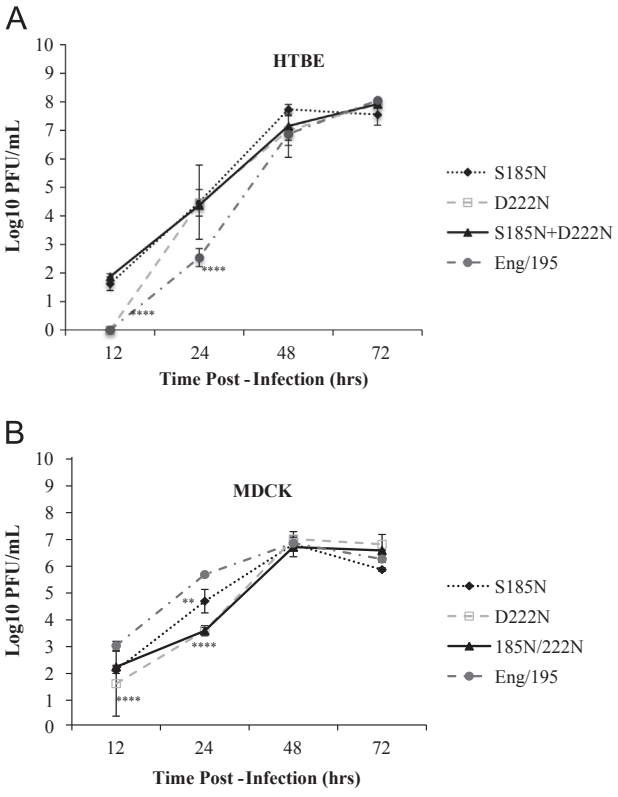



**Fig. 1.** Replication of viruses in cell culture. Growth kinetics of WT and mutated viruses in (A) HTBE and (B) MDCK cultures. Cells were infected at an MOI of 0.001 or 0.01. Data represent the average ± SD of three different experiments. Statistics were calculated with the ANOVA test for repeated measures using GraphPad Prism v5.0. Significant differences were considered $^{****}p < 0.0001$; $^{**}p < 0.001$.

Notably, in the MDCK cells, recombinant viruses containing the D222N polymorphism (rEng/195-D222N and rEng/195-S188N+D222N) showed a reduction in viral replication, and we speculate that this likely reflects differences in cell surface receptor composition compared to the HTBE cell.

*Receptor binding profile of A(H1N1)pdm09 virus with D222N and secondary mutations at the Sb antigenic site*

To identify potential differences in receptor recognition among the viruses, we analyzed their binding properties using a carbohydrate microarray. Overall, the three mutant viruses displayed fairly similar binding properties compared to the wild type virus (Fig. 2A–D). All recombinant viruses showed significant binding to the α2,6 glycan #481 with a bifurcated structure, and binding peaks were also observed to the glycan #331 with a linear structure, though only for the rEng/195-wt and single mutant rEng/195-S185N (Fig. 3A–D) was this considered significant (two times the background average, with a % CV of less than 50%). A similar result was observed for the glycan #11 (Fig. 2A, C and D) that consists in a single molecule of SA (Neu5Ac), with significant binding for the rEng/195-wt, rEng/195-D222N and the double mutant rEng/195-S185N+D222N. Differences in sialic acid linkage specificity of mutant viruses were also observed. The rEng/195-wt virus bound significantly to a wide number of α2,6 glycans but also to a significant number of α2,3 in agreement to previous reports (Bradley et al., 2011; Chen et al., 2011). The rEng/195-D222N bound significantly to α2,6 glycans (#331, 481, 601 and 605) but also bound to the α2,3-linked glycan #236. The weak binding to α2,3 glycans shown by the rEng/195-D222N virus has been previously address with similar results using a recombinant virus with a D222N mutated HA (Yang et al., 2010). The rEng/195-S185N bound to a wider range of α2,6 glycans (#331, 481, 601, 605, 55, 57, 501, 344 and 600) and showed no significant binding to α2,3 glycans. Notably, the double mutant rEng/195-S185N+D222N showed significant binding to both α2,3 (#599, 606, 460) and α2,6 glycans (#481, 479).

Interestingly, all three mutants exhibited prominent binding to non-Neu5Ac glycans of long bi-or tri-antennary structure containing repeated molecules of galactose (Gal) and N-acetyl-glucosamine (GlcNac), some of them containing a fucose group (Fig. 2B–D). It is also notable that the preference for these non-sialylated glycans is enhanced when the mutation S185N is added to the single mutant D222N. The structure of the top five glycans with significant binding for each virus is shown in Fig. 3A–D. Notably, a similar pattern of binding has been reported for an A(H1N1)pdm09 virus isolated in a fatal case of infection from a pediatric patient (Bradley et al., 2011), though, based on the reported partial sequence of the HA protein, this virus differs at amino acid 185 and 222 compared to our mutants. The significance of these findings is unclear.

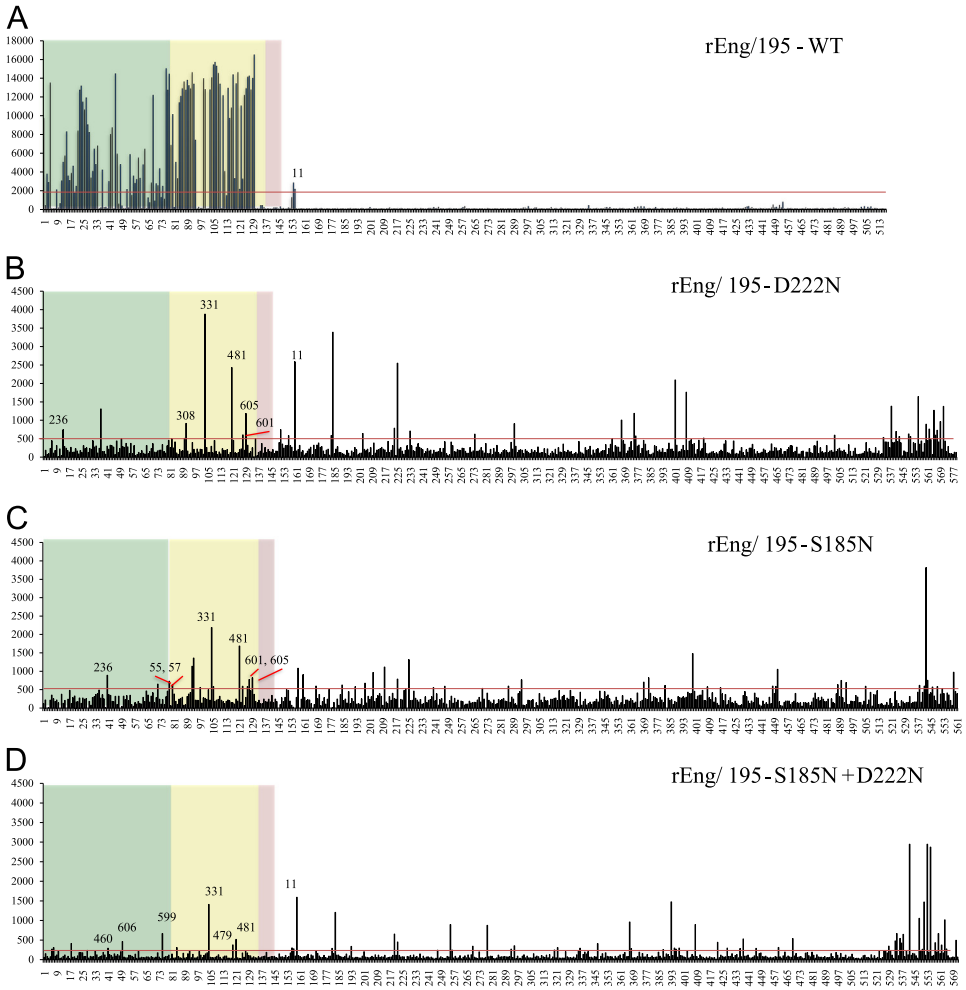
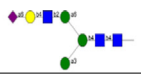
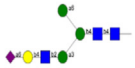
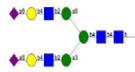



**Fig. 2.** Glycan microarray analysis. Receptor binding specificity of (A) wild type and (B–D) mutant viruses. Glycans were reordered from the original format as follows: green, α2,3 sialosides; yellow, α2,6 sialosides; pink, α2,8 sialosides; no color, sialic acid and asialo-glycans. The number of glycans on the abscissa corresponds only to glycans with RFU values above zero. The fluorescent binding signal intensity is indicated at the *y* axis. The red line across the graph corresponds to the background average in RFU: 2270 for rEng/195-wt; 512 for rEng/195-D222N; 569 for rEng/195-S185N; and 290 for rEng/195-S185N+D222N.

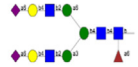
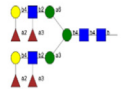
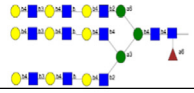
A

rEng/195 -wt

| Glycan# | Glycan Structure | Fold above background |
|---|---|---|
| 345 |  | 7.2 |
| 346 |  | 6.8 |
| 331 |  | 6.7 |
| 347 |  | 6.7 |
| 55 |  | 6.6 |

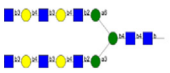
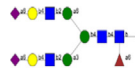
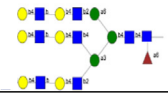
B

rEng/195 -D222N

| Glycan # | Glycan Structure | Fold above background |
|---|---|---|
| 35 |  | 6.6 |
| 11 |  | 5.0 |
| 481 |  | 4.7 |
| 361 |  | 3.4 |
| 570 |  | 3.2 |

C

rEng/195 -S185N

| Glycan # | Glycan Structure | Fold above background |
|---|---|---|
| 545 |  | 6.7 |
| 331 |  | 3.8 |
| 481 |  | 2.9 |
| 82 |  | 2.3 |
| 581 |  | 1.9 |

D

rEng/195-S185N+D222N

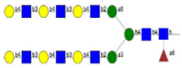
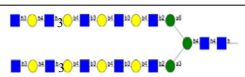
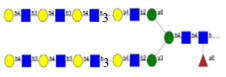
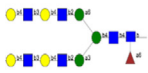
| Glycan# | Glycan Structure | Fold above background |
|---|---|---|
| 576 |  | 10.1 |
| 563 |  | 10.1 |
| 578 |  | 9.9 |
| 11 |  | 5.4 |
| 574 |  | 5.1 |

**Fig. 3.** Structure of the top highest binding glycans. Top five glycans with %CV less than 50% bound by (A) wild type and (B–D) mutant viruses. %CV was provided by glycan array analysis software. Glycan nomenclature is as follows: purple diamond, Neu5Ac; yellow circle, Galactose; yellow square, GalNac; blue square, GlcNAc; red triangle, fucose; $OSO_3$, sulfate group; green circle, Mannose. Linkages are indicated between sugar symbols.

*Secondary mutation S185N modifies the cell tropism of D222N virus by increasing the infection rate in HTBE culture*

Finally, we investigated the cell tropism and infection rate of the recombinant viruses in HTBE cells. Infected HTBE sections were stained with antiserum to detect influenza NP, and co-stained with lectins, *Maackia amurensis* (MAL I) or *Sambucus nigra* (SNA; Fig. 4A). The number and type of infected cells was recorded from three different sections. Absolute numbers from cell counts (Fig. 4B) indicate that mutants rEng/195-S185N and rEng/195-S185N+D222N infected the highest number of cells, followed by the rEng195-wt. Interesting, the single mutant rEng195-D222N infected the lowest percentage of HTBE cells, three times less than the virus containing the double mutant HA. These results are in agreement with the data from the growth curve; it confirms our previous observation that mutation D222N affects early infection rates within the first 24 h post-infection, reducing the number of infected cells.

Co-staining for viral antigen and sialic acids (Fig. 4C) revealed that most of the cells (70%) infected by rEng/195-wt also stained for SNA, indicating they expressed α2,6-SA. Our previous data suggests many of these are non-ciliated cells (Ayora-Talavera et al., 2009; Thompson et al., 2006). The single mutant rEng/195-D222N also showed preferential infection of cells expressing α2,6-SA. However, the single mutant rEng/195-S185N or double mutant rEng/195-S185N+D222N infected cells co-stained for SNA or MAA equivalently, suggesting the increase in number of cells infected was mediated by an ability to enter cells using either α2,6-SA or α2,3-SA receptors. Taken together with the finding of increased replication in HTBE, these data indicate that the single mutation S185N confers efficient recognition of both types of receptors, and that even when coupled with D222N, it maintains the broad tropism phenotype. We suggest that the combination S185N+D222N detected in a fatal influenza infection led to increased replication and expanded cell tropism.

The observations of heterogeneity at positions such as HA1 222 encompassing residues that convey different binding phenotypes might reflect a balance between selective pressures operating on these virus populations. Transmission efficiency in humans would favor viruses with α2,6-SA specificity due to the abundance of α2,6-SA in the upper respiratory tract of humans, and resistance to inhibitory effects of α2,3-SA-rich respiratory mucins. However, within infected individuals selection of α2,3-SA binding variants could allow for attachment to alternative cell types. Enrichment of the latter phenotype might be expected in patients displaying more severe symptoms, including infection of the lower respiratory tract where α2,3-SA receptors are more prevalent.

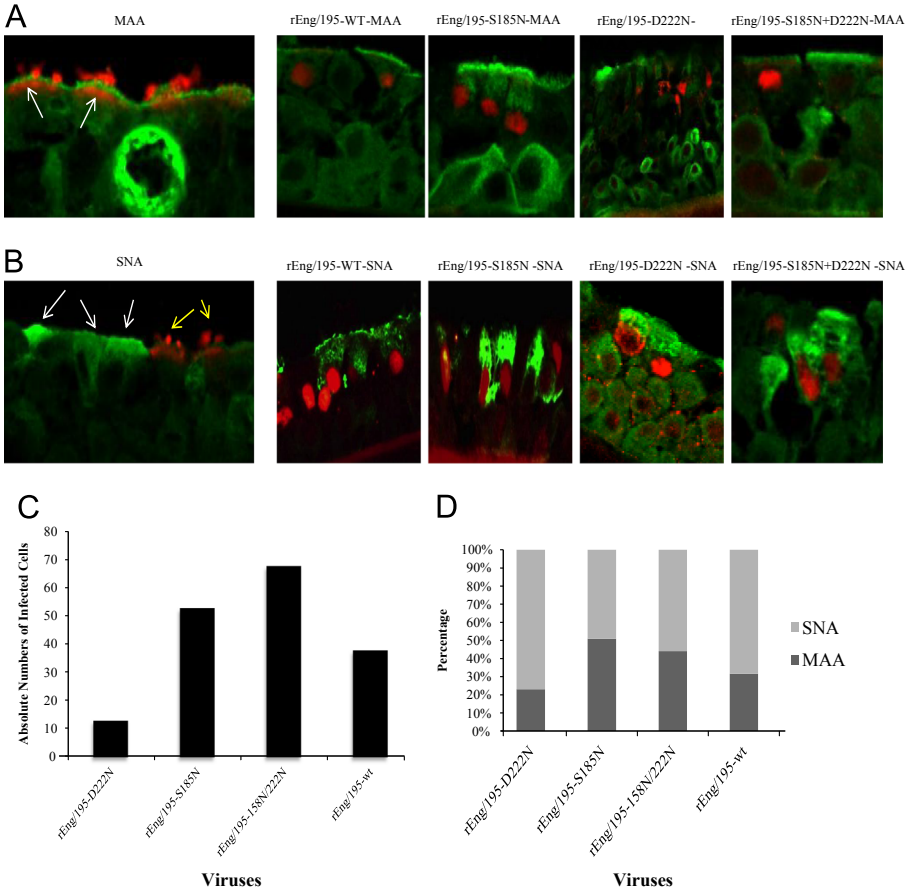



**Fig. 4.** Binding and cell tropism in HTBE cultures. Lectin binding and co-staining with anti-influenza NP. The preference of binding to ciliated and non-ciliated cells by lectins (A) MAA and (B) SNA is shown by white arrows respectively. Yellow arrows indicate cilia stained with anti-alpha tubulin. The (A) right panels show co-staining between MAA or SNA and influenza NP protein for rEng/195-WT and each mutant. (B) The total number of infected cells (nucleus stained in red) for each virus was counted from two different tissue sections. (C) The preference of infection and cell tropism of the WT and mutant viruses was inferred from the co-staining with SNA and MAA lectins; data are expressed as percentage considering the absolute numbers of infected cells as the 100%.

## Conclusion

We have described the receptor binding properties of an A (H1N1)pdm09 virus containing a D222N substitution at a position noted for polymorphisms in clinical isolates from severe or fatal influenza. Our results suggest that viruses containing this mutation alone may not contribute to the severity/fatality of the affected patients as indicated by the loss of binding and delayed replication in HTBE cultures.

The literature review indicates that substitution D222N is detected as a minor population in viruses isolated from throat and/or bronchial samples (Baldanti et al., 2011; Resende et al., 2014; Wang et al., 2011); worldwide the D222N has been reported in only 0.41% of viral sequences (http://www.flusurver.bii.a-star.edu.sg). In Mexico, polymorphisms 222R/G/N are found in 7.7% of 361 HA sequences; where the frequency of detection is 53% for 222R [code for A or G-AAT (N), AGT (S), GGT (G) or GAT (D)], 36% for 222G and 11% for 222N. The clinical and epidemiological analysis for some of these viruses has been recently described (Vazquez-Perez et al., 2013).

The most interesting and novel finding of our study is the effect that S185N has on the binding, replication and cell tropism of the A(H1N1)pdm09 virus. In public databases, this mutation has been reported only in a small proportion of viral isolates (0.34%), and to our knowledge, this is the first report of the effects of S185N in conjunction with D222N.

In conclusion, in this study we have addressed the impact of viral variants with mutations that affect virus survival at early time during replication. These viruses evolve by acquiring compensatory mutations to expand their cell tropism and, as a consequence, increase the severity of the infection and clinical outcome of the patient (Casalegno et al., 2014; Houng et al., 2012; Vazquez-Perez et al., 2013).

## Materials and methods

### *Cell lines*

Madin-Darby Canine Kidney (MDCK) cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Gibco, USA) and 1% of non essential amino acids 100x (Sigma-Aldrich, USA). Cells were incubated at 37 °C in 5% $CO_2$.

Human tracheobronchial epithelial (HTBE) cells were obtained from MatTek (Massachusetts, USA) and shipped ready to use in 24 wells plate and seeded on transwell membrane of 6.5 mm of diameter with a pore size of 0.4 μm (Corning, USA). HTBE cells were cultured following manufacturer's protocol. Briefly, HTBE cells were grown in an air–liquid interface during 20 days to form a differentiated, polarized culture that resemble in vivo the pseudostratified mucociliary epithelium. AIR-100-ASY pre-warmed media was replaced in the basal area of the culture every 72–96 h. Prior to the experiment, cells were rinsed with AIR-100-ASY media and incubated during 30 min at 37 °C, 5% $CO_2$ to remove the excess mucus.

*Viruses*

Recombinant viruses were generated by reverse genetics on the background of the viral A(H1N1)pdm09 virus A/England/195/2009 (rEng195). Viruses were rescued by a 12 plasmid transfection of 293T and MDCK co-culture as previously described (Shelton et al., 2012). Relevant mutations were generated by direct mutagenesis with Quickchange Lightning Site-Directed Mutagenesis protocol (Stratagene, CA, USA). A total of four recombinant viruses including the WT were rescued, viruses containing single S185N, D222N and double mutations S185N+D222N at the RBS of the HA were identified as rEng195-WT, rEng195-S185N, rEng195-D222N and rEng195-S185N+D222N, respectively. The HA gene of recombinant viruses was sequenced to detect the emergence of possible revertants and subdominant sequence variants during amplification.

*Cell infection*

MDCK cells were infected with mutant rEng195-S185N, rEng195-D222N and rEng195-S185N+D222N and rEng195-WT viruses at multiplicity of infection (MOI) of 0.01 PFU. After 1 h incubation at room temperature (RT), viruses were removed and cells incubated for 72 h at 37 °C with DMEM supplemented with 2 μg/ml of TPCK-Trypsin. Viruses were harvested every 12 h and viral titer was obtained by standard plaque assay in MDCK cells.

HTBE cells were infected with viruses at MOI of 0.001 PFU. After 1 h incubation at RT, viruses were removed and cells incubated for 72 h at 37 °C with MucilAir media.

Prior to virus collection, cells were incubated for 30 min with 330 μl of media; secreted viruses were harvested at 12, 24, 48 and 72 h post-infection from the apical side of the culture. Viral titer was obtained by plaque assay in MDCK cells.

*Virus purification and labeling*

Prior to analysis on the glycan array, viruses were grown in MDCK cells and purified by ultra-speed centrifugation through a 25% sucrose cushion in NTE buffer (100 mM Tris–HCl pH7.4, 100 mM NaCl and 1 mM EDTA). Viruses were centrifuged for 3 h at 25,000 rpm at 4 °C, supernatant was removed and viruses were incubated in 250–500 μL of PBS 1× overnight. Viruses were resuspended, aliquoted and stored at −80 °C.

Viruses were labeled with the amine reactive dye Alexa Fluor488 (Invitrogen, USA). Twenty-five micrograms of amine reactive Alexa Fluor488 was incubated with 300 μL of virus and 30 μL of 1 M NaHCO3 (pH 9.0) for 1 h. The labeled viruses were dialyzed overnight in a 7000 MWCO Slide-A-Lyzer MINI dialysis unit (Thermo Scientific) against PBS+1 mM EDTA.

*Glycan arrays*

The Consortium of Functional Glycomics performed glycan microarray binding experiments (www.functionalglycomics.org) using Core H, version V5.2. Significant peaks were determined by averaging the relative fluorescent units (RFU) of all glycans on the microarray, which was then multiplied by two to determine the background RFU level. Significant binding was considered to be two times the background average, with a % CV of less than 50%.

*Immunohistochemical analysis*

Human tracheobronchial epithelial cells (HTBE) cells were infected with mutants and WT viruses at multiplicity of infection (MOI) of 1.0 and allowed to incubate for 16 h at 37 °C. Transwell membranes were embedded in paraffin by standard tissue processing procedures, and cut at 4 μm and affixed on glass slides in collaboration with the Yerkes National Primate Research Center at Emory University. Lectin staining was performed using biotin labeled *Sambucus nigra* (SNA) and *Maackia amurensis* agglutinins (MAA), purchased from Vector Laboratories (USA). The sections were deparaffinized and rehydrated. Non-specific binding was blocked using 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 16 h at 4 °C before incubation with ligands. SNA or MAA were diluted in 1% BSA in PBS and incubated 16 h at 4 °C. After washing in PBS-T, slides were incubated with complexed Streptavidin-FITC (Invitrogen) in 1% BSA/PBS. For co-localization of influenza A proteins, slides were stained with mouse anti-influenza A nucleoprotein (NP) and goat anti-mouse IgG conjugated to Alexa Fluor 568 (Invitrogen) diluted in 1% BSA/PBS for 1 h at 25 °C. Slides were washed and viewed under a confocal microscope. Images were collected on a Zeiss Pascal LSM510 laser scanning microscope using Axiovert2 imaging software using a Plan-Apochromat 40 × 1.4 Ph3 lens. The number of different cell types infected by each virus was quantified by counting at least 10 different fields of two different tissue sections.

## Aknowledgments

This work was supported by Consejo Nacional de Ciencia y Tecnología Grant SEP/Conacyt-2009-131965. Confocal work was performed in LANNBIO-Cinvestav Merida (CONACYT Proy Lab 123913 y FOMIX-Yucatán Proy 108160). We thank MSc. Heredia-Cervera for the technical assitance. LBL and DAS acknowledge support by the U.S. Department of Health and Human Services contract HHSN272201400004C (NIAID Centers of Excellence for Influenza Research and Surveillance).

## References

Abed, Y., Pizzorno, A., Hamelin, M.E., Leung, A., Joubert, P., Couture, C., Kobasa, D., Boivin, G., 2011. The 2009 pandemic H1N1 D222G hemagglutinin mutation alters receptor specificity and increases virulence in mice but not in ferrets. J. Infect. Dis. 204, 1008–1016.

Ayora-Talavera, G., Shelton, H., Scull, M., Ren, J., Jones, M.J., Pickles, R.J., Barclay, W.S., 2009. Mutations in H5N1 influenza virus hemagglutinin that confer binding to human tracheal airway epithelium. PLos One 4, e7836.

Ayora-Talavera, G., Betancourt-Cravioto, M., Gómez-Carballo, J., Conde-Ferráez, L., González-Losa, R., Manrique-Saide, P., Cuauhtémoc Sánchez, E., Quijano-Vivas, A., 2012. Epidemiologic study of human infl uenza A (H1N1) pdm09 virus in Yucatan, Southern Mexico. Rev. Biomed. 23, 39–46.

Ayora-Talavera, G., Cetina-Montejo, L., Matos-Patrón, A., Romero-Beltrán, L., 2014. Hemagglutinin variants of influenza A(H1N1)pdm09 virus with reduced affinity for sialic acid receptors. Arch. Virol. 159, 1207–1211.

Baillie, G.J., Galiano, M., Agapow, P.M., Myers, R., Chiam, R., Gall, A., Palser, A.L., Watson, S.J., Hedge, J., Underwood, A., Platt, S., McLean, E., Pebody, R.G., Rambaut, A., Green, J., Daniels, R., Pybus, O.G., Kellam, P., Zambon, M., 2012. Evolutionary dynamics of local pandemic H1N1/2009 influenza virus lineages revealed by whole-genome analysis. J. Virol. 86, 11–18.

Baldanti, F., Campanini, G., Piralla, A., Rovida, F., Braschi, A., Mojoli, F., Iotti, G., Belliato, M., Conaldi, P.G., Arcadipane, A., Pariani, E., Zanetti, A., Minoli, L., Emm, V., 2011. Severe outcome of influenza A/H1N1/09v infection associated with 222G/N polymorphisms in the haemagglutinin: a multicentre study. Clin. Microbiol. Infect. 17, 1166–1169.

Bradley, K.C., Jones, C.A., Tompkins, S.M., Tripp, R.A., Russell, R.J., Gramer, M.R., Heimburg-Molinaro, J., Smith, D.F., Cummings, R.D., Steinhauer, D.A., 2011. Comparison of the receptor binding properties of contemporary swine isolates and early human pandemic H1N1 isolates (Novel 2009 H1N1). J. Virol. 413, 169–182.

Casalegno, J.S., Ferraris, O., Escuret, V., Bouscambert, M., Bergeron, C., Line's, L., Excoffier, T., Valette, M., Frobert, E., Pillet, S., Pozzetto, B., Lina, B., Ottmann, M., 2014. Functional balance between the hemagglutinin and neuraminidase of influenza A(H1N1)pdm09 HA D222 variants. PLos One 9, e104009.

Chen, C., Wen, X., To, K.K.W., Wang, P., Tse, H., Chan, J.F.W., Tsoi, H.W., Fung, K.S.C., Tse, C.W.S., Lee, R.A., Chan, K.H., Yuen, K.Y., 2010. Quasispecies of the D225G substitution in the hemagglutinin of pandemic influenza A(H1N1) 2009 virus from patients with severe disease in Hong Kong, China. J. Infect. Dis. 201, 1517–1521.

Chen, LM, Rivailler, P., Hossain, J., Carney, P., Balish, A., Perry, I., Todd Davis, C., Garten, R., Shu, B., Xu, X., Klimov, A., Paulson, J.C., Cox, N., Swenson, S., Stevens, J., Vincent, A.,

Gramer, H., Donis, R.O., 2011. Receptor specificity of subtype H1 influenza A viruses isolated from swine and humans in the United States. Virology 412, 401–410.
Chowell, G., Bertozzi, S., Coclhero, A., Lopez-Gatel, H., Alpuche-Aranda, C., Hernandez, M., Miller, M.A., 2009. Severe respiratory disease concurrent with the circulation of H1N1 influenza. N. Engl. J. Med. 361, 674–679.
Chowell, G., Echevarría-Zuno, S., Viboud, C., Simonsen, J., Tamerius, M., Miller, V., et al., 2011. Characterizing the epidemiology of the 2009 influenza A/H1N1 pandemic in Mexico. PLoS Med. 8, e1000436.
Chutinimitkul, S., Herfst, S., Steel, J., Lowen, A.C., Jianqiang, Y., van Riel, D., et al., 2010. Virulence-associated substitution D222G in the hemagglutinin of 2009 pandemic influenza A(H1N1) virus affects receptor binding. J. Virol. 84, 11802–11813.
de Vries, R.P., de Vries, E., Martínez-Romero, C., McBride, R., van Kuppeveld, F.J., Rottier, P.J.M., García-Sastre, A., Paulson, J.C., de Hann, C.A.M., 2013. Evolution of the hemagglutinin protein of the new pandemic H1N1 influenza virus: maintaining optimal receptor binding by compensatory substitutions. J. Virol. 87, 13868–13877.
Drews, S.J., Pabbaraju, K., Wong, S., Tokaryk, K.L., May-Hadford, J., Lee, B., Tellier, R., Louie, M., 2011. Surveillance of autopsy cases for D222G substitutions in haemagglutinin of the pandemic (H1N1) 2009 virus in Alberta, Canada. Clin. Microbiol. Infect. 17, 582–584.
ECDC. 2013. Influenza virus characterization. Surveillance report. Summary Europe December 2013.
El Moussi, A., Kacem, M.A.B., Pozo, F., Ledesma, J., Cuevas, M.T., Casas, I., Slim, A., 2013. Frequency of D222G haemagglutinin mutant of pandemic (H1N1) pdm09 influenza virus in Tunisia between 2009 and 2011. Diagn. Pathol. 8, 124.
Elderfield, R., Watson, S.J., Godlee, A., Adamson, W.E., Thompson, C.I., Dunning, J., Fernandez-Alonso, M., Blumenkrantz, D., Hussell, T., The MOSAIC investigators, Zambon, M., Openshaw, P., Kellam, P., Barclay, W.S., 2014. Accumulation of human-adapting mutations during circulation of A(H1N1)pdm09 influenza in humans in the UK. J. Virol. 88, 13269–13283.
Houng, H.S.H., Garner, J., Zhou, Y., Lyons, A., Kuschner, R., Deye St, G., Clair, K., Douce, R.W., Chicaiza, W., Blair, P.J., Myers, C.A., Burke, R.L., Sanchez, J.L., Williams, M., Halsey, E.S., 2012. Emergent 2009 influenza A(H1N1) viruses containing HA D222N mutation associated with severe clinical outcomes in the Americas. J. Clin. Virol. 53, 12–15.
Klimov, A,G.R., Russell, C., Barr, I.G., Besselaar, T.G., Daniels, R., Engelhardt, O.G., Itamura, S., Kelso, A., McCauley, J., Odagiri, T., Smith, D., Tashiro, M., Xu, X., Webby, R., Wang, D., Ye, Z., Yuelong, S., Zhang, W., Cox, N., Writing Committee of the World Health Organization Consultation on Southern Hemisphere Influenza Vaccine Composition for 2012, 2012. WHO recommendations for the viruses to be used in the 2012 southern hemisphere influenza vaccine: epidemiology, antigenic and genetic characterization of influenza A(H1N1) 9pdm09, A(H3N2) and B influenza viruses collected from February to September 2011. Vaccine 30, 6461–6471.
Ledesma, J., Pozoa F., Ruiz M.P., Navarro J.M., Piñeiroc L., Montes M., Castro S.P., Fernández J.F., Costa J.G., Fernández M., Galáng J.C., Cuevas M.T., Casas I., Breña P.P., Spanish Influenza Surveillance System (SISS). 2011. Substitutions in position 222 of haemagglutinin of pandemic influenza A (H1N1) 2009 viruses in Spain. J. Clin. Virol. 51:75-78.
Liu, Y., Childs, R.A., Matrosovich, T., Wharton, S., Palma, A.S., Chai, W., Daniels, R., Gregory, V., Uhlendorf, J., Kiso, M., Klenk, H.D., Hay, A., Feizi, T., Matrosovich, M., 2010. Altered receptor specificity and cell tropism of D225G hemagglutinin mutants isolated from fatal cases of pandemic A(H1N1) 2009 influenza virus. J. Virol. 84, 12069–12074.
Matrosovich, M., Matrosovich, T.Y., Gray, T., Roberts, N.A., Klenk, H.D., 2004. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc. Natl. Acad. Sci. 101, 4620–4624.
Resende, P.C., Motta, F.C., Oliveira, M.L.A., Gregianini, T.S., Fernandes, S.B., Cury, A.L.F., Rosa, M.C.D., Souza, M.L., Siqueira, M.M., 2014. Polymorphisms at residue 222 of the hemagglutinin of pandemic influenza A(H1N1)pdm09: association of quasispecies to morbidity and mortality in different risk categories. PLos One 9, e92789.
Rykkvin, R., Kilander, A., Dudman, S.D., Hungnes, O., 2013. Within-patient emergence of the influenza A(H1N1) pdm09 HA1 222G variant and clear association with severe disease, Norway. Euro Surveill 18, 20369.
Shelton, H., Smith, M., Hartgroves, L.C.S., Stilwell, P., Roberts, K.L., Johnson, B., Barclay, W.S., 2012. An influenza reassortant with polymerase of pH1N1 and NS gene of H3N2 influenza A virus is attenuated in vivo. J. Gen. Virol. 93, 998–1006.
Shinya, K., Ebina, M., Yamada, S., Ono, M., Kasai, N., Kawaoka, Y., 2006. Influenza virus receptors in the human airway. Nature 440, 435–436.
Thompson, C.I., Barclay, W.S., Zambon, M.C., Pickles, R.J., 2006. Infection of human airway epithelium by human and avian strains of influenza A virus. J. Virol. 80, 8060–8068.
Van Riel, D., Munster, V.J., de Wit, E., Rimmelzwaan, G.F., Fouchier, R.A.M., Osterhaus, ADME, Kuiken, T., 2006. H5N1 virus attachment to lower respiratory tract. Science 312, 399.
Vazquez-Perez, J., Isa, P., Kobasa, D., Ormsby, C.E., Ramírez-Gonzalez, J.E., Romero-Rodríguez, D.P., Ranadheera, C., Li, Y., Bastien, N., Embury-Hyatt, C., González-Duran, E., Barrera-Badillo, G., Ablanedo-Terrazas, Y., Sevilla-Reyes, E., Escalera-Zamudio, M., Cobián-Güemes, A., Lopez, I., Ortiz-Alcántara, J., Alpuche-Aranda, C., Perez-Padilla, J., Reyes-Terán, G., 2013. A (H1N1) pdm09 HA D222 variants associated with severity and mortality in patients during a second wave in Mexico. Virol. J. 10, 41.
Wang, B., Dwyer, D.E., Soedjono, M., Shi, H., Matlho, K., Ratnamohan, M., Blyth, C., McPhie, K., Cunningham, A.L., Saksena, N.K., 2011. Evidence of the circulation of pandemic influenza (H1N1) 2009 with D222D/G/N/S hemagglutinin polymorphisms during the first wave of the 2009 influenza pandemic. J. Clin. Virol. 52, 304–306.
Xu, R., Ekiert, D.C., Krause, J.C., Hai, R., Crowe Jr, J.E., Wilson, I.A., 2010. Structural basis of preexisting immunity to the 2009 H1N1 pandemic influenza virus. Science 328, 357–360.
Yang, H., Carney, P., Stevens, J., 2010. Structure and Receptor binding properties of a pandemic H1N1 virus hemagglutinin. PLOS Currents Influenza, http://dx.doi.org/10.1371/currents.RRN1152.